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## 5-INTRODUCTION

Homeobox genes (HOX) are a family of regulatory genes encoding a closely related subset of homeobox containing transcription factors that primarily play a crucial role in embryogenesis. The homeobox of these factors encodes a 61 amino acid homeodomain that binds specifically to DNA. After embryogenesis, HOX genes may continue to be transcribed according to a tissue-specific pattern of expression. To date, 39 class I homeobox genes have been identified in human. They are organized in four clusters (HOX A, B, C and D), located respectively on chromosomes 7, 17, 12 and 2 [1,2]. Several homeobox-containing genes have been observed to display an altered pattern of expression in some malignancies when compared with the corresponding normal tissues [3,4]. Moreover, misregulation of certain homeobox genes can lead to cellular transformation in culture, as well as tumor formation in vivo [5-7]. There is evidence that some of the HOX genes involved in the development of various solid tumors including human breast cancer [8,9]. These observations suggest that in addition to their role in embryogenesis, homeobox-containing genes may play an important role both in controlling cell differentiation and in the multistep process of tumorigenesis. However, few data are presently available on HOX gene regulation in normal or malignant human breast epithelial cells. It is known that some peptide growth factors activate the expression of homeobox genes through a cystine-knot-folding motif. Human chorionic gonadotropin (hCG) is a glycoprotein hormone containing a cystine-knot folding motif that suggest that it also could interact with a homeodomain. HCG is the first hormone secreted by the extra embryonic tissue and therefore it is possible to postulate that it could be an important developmental factor. In our previous studies, hCG has been proven to be an efficacious physiological protector in rats against the initiation and progression of mammary tumors induced by carcinogen 7, 12-dimethylbenz(a)anthracene (DMBA), accompanied by the induction of cellular differentiation [10,11], depression of the proliferation of breast tumor cells *in vitro* and activation of programmed cell death [12,13]. A recent report indicates that hCG could affect the tumor necrosis factor (TNF)-induced activation of the activator protein (AP)-1 [14]. Altogether these data suggested that hCG effect on the mammary epithelial cells could be mediated by HOX gene expression, probably through inhibiting/activating AP-1 transcription activities that in turn regulates genes responsible for critical events in cell differentiation such as, DNA repair, apoptosis and cell transformation.

The original three-year award requested was funded for only two years. Therefore we have decided to concentrate our efforts in the effect of r-hCG and to identify possible targets of the HOX genes by using cDNA array analysis. During this two-year award we have been able to accomplish the following:

- 1-to perform a comprehensive survey of class I homeobox genes expression in an immortalized human breast epithelial cell line, MCF-10F, and the human breast cancer-derived estrogen receptor (ER)-positive cells MCF7 as well as ER-negative cells MDA-MB-231.

- 2-to determine the level of expression of these HOX genes after treatment of these human breast epithelial cells with recombinant hCG (r-hCG) at various length of time.

- 3- to demonstrate that HOXA1 has a divergent expression and induction by hCG in human breast epithelial cells.

- 4- to demonstrated that hCG- modulate genes that encode transcription factors/DNA-binding proteins using cDNA array techniques.

- 5-. to demonstrate that r-hCG-modulates the expression of HOX2, a silent gene in MCF10F cells,

showing its role in activation of AP-1 transcription factor.

## **6-BODY**

### **6-A-Methods and Procedures:**

#### ***Cell Culture and Treatment:***

The spontaneously immortalized human breast epithelial cells MCF-10F, which originated from the mortal cells MCF-10M [15,16], human breast cancer-derived MCF-7 cells and MDA-MB-231 which both had been obtained from the ACTT (Rockville, MD), were maintained in our laboratory. MCF-10F cells were grown in DMEM/F12 medium supplemented with 5% horse serum, insulin, hydrocortisone, epidermal growth factor, cholera toxin, and antibiotics. MCF-7 and MDA-MB-231 cells were grown in DMEM/F12 medium supplemented with 10% fetal calf serum, insulin, and antibiotics. r-hCG was obtained from Serono, Norwell, MA. Cells were treated with r-hCG at 5 µg/ml in appropriate media for 1, 5, 10, 24, 48, and 96 hours. Control cells were treated with same volume of buffer in which the r-hCG was dissolved. The culture media with or without the hormone were replaced daily for those cells who were for 48 and 96-hour in culture.

#### ***RNA Extraction:***

The cells were harvested at the special time intervals mentioned above, and immediately frozen in liquid nitrogen following wash in cold PBS. For extraction of total RNA, the frozen MCF-10F, MCF-7 and MDA-MB-231 cells were homogenized in TRIzol Reagent (Gibco BRL, Gaithersburg, MD). The RNA was isolated according to the manufacturer's instructions and stored in RNase-free water at -70 °C. The integrity of total RNA was determined by analyzing on agarose gel.

#### ***Reverse Transcription-polymerase Chain Reaction (RT-PCR).***

RT-PCR was performed by using QIAGEN OneStep RT-PCR Kit (Qiagen Inc., Valencia, CA). To evaluate the expression level of HOX genes, an aliquot of total RNA (800ng/50µl) was used for each reaction. Reverse transcriptions were performed at 50°C for 35 min. PCR were carried out with a 15 min hot start at 95°C followed by a denaturation step at 94°C for 45 sec, an annealing step at the appropriate temperature for 45 sec and an extension step at 72°C for 45 sec for 35-40 cycles [18,19]. Each reaction was finished by a final extension run at 72°C for 10 min. Specific primers for each gene of the clusters A, B, C and D were used as depicted in Table 1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was used as an internal control of expression using following primers: 5' primer, 5'-ACATCAAGAAGGTGGTGAAGCAGG-3', 3' primer, 5'-CTCTTCCTCTTGTGCTCTTGCTGG-3'. The reverse transcription procedure was the same that in HOX genes detection, but the amplification was achieved by 20 PCR cycles involved 1 min at 94°C for denaturation, 1 min at 56°C for annealing and 1min at 72°C for extension.

#### ***Sequence Analysis:***

Fresh RT-PCR products containing the bands of interesting in gel were directly cloned to pCR®4-TOPO vector (Invitrogen Corporation, Carsbad, CA 92008) and submitted for automatic DNA sequence analysis in the DNA Sequence Facility of Fox Chase Cancer Center (Philadelphia, PA). Computer program the

Baylor College of Medicine Search Launcher was used to analyze the sequence data and identify the mRNA isoforms.

### ***Semi-quantitative Analysis:***

The PCR products were electrophoresed in 2% agarose gel and stained with ethidium bromide. The images were captured by a Polaroid MP-4 Land Camera. Semi-quantitative analysis for the expression levels based on the gel band density calculation comparing to the samples without hCG treatment (controls) and normalized by GAPDH gene expression levels by means of Computing Densitometer (Molecular Dynamics Co., USA).

### ***Construction of HOXA2 Expression Vector, Transient Transfection and TNF Treatment of MCF-10F cells.***

The cDNA encoded HOXA2 amino acid 183-376 was generated from human PAC clone DJ0167F23 (purchase from Research Genetics Inc., Huntsville, AL) by *Taq*-amplified PCR and was subcloned into the mammalian expression vector pCMV-Tag4 (Stratagene Inc., La Jolla, California). Sequences were analyzed to confirm that the insert was in the correct reading frame. Transfections in MCF-10F cells were performed using Lipofectamine Reagent (GIBCOBRL, Rockville, MD) for 1 $\mu$ g of pCMV-HOXA2 plasmids per 35-mm dish following the user's instruction. After 48 hours of the start transfection, the cells were treated with 10 ng/ml of RANK ligand (a ligand of TNF receptor family) for 1, 12, or 24 hours, respectively.

### ***Electrophoretic Mobility Shift Assay (EMSA).***

Nuclear proteins were isolated using a method described previously [17], with small modifications. Briefly, the cells were rinsed with ice cold PBS (Ca<sup>2+</sup> and Mg<sup>2+</sup> free) and scraped from the dishes. Resuspended the cells in 400  $\mu$ l of buffer A (25 mM HEPES, 10 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.5 mM EDTA) containing 10% NP-40 and protease inhibitors (50mM DTT, 0.2 M PMSF, 10  $\mu$ g/ml leupeptin ). Following a 10 min incubation, the supernatant was removed and the nuclei resuspended in 50  $\mu$ l of buffer B (20 mM HEPES, 0.42M NaCl, 5 Mm EDTA, 10% glycerol) plus protease inhibitors as above. Vortexed vigorously for 3 min and kept in a shaking platform for 30 min. The supernatants were collected and the concentration was determined using the Bio-Rad protein assay kit (Bio-Rad Labs, Hercules, CA). Gel shift assay system was purchased from Promega Corporation (Madison, WI). The oligonucleotides corresponding to an AP-1 site (5'-CGCTTGATGAGTCAGCCGGA-3') was radiolabeled using  $\alpha$ -<sup>32</sup>P-dCTP and T4 polynucleotide kinase. For the binding reaction, <sup>32</sup>P-labeled oligonucleotide (20,000-25,000 cpm) was incubated with 3 $\mu$ g of nuclear extract and gel shift binding buffer [4% glycerol, 1mM MgCl<sub>2</sub>, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris-HCl pH7.5, 50 $\mu$ g/ml poly(dI-dC) • poly(dI-dC)] at room temperature for 20 min. DNA-protein complexes were subjected to electrophoresis on a 4% polyacrylamide gel with 0.5 $\times$ TBE running buffer (90 mM Tris, 90 mM boric acid, 2 mM EDTA pH8.0) at 11 V/cm. The gels were dried on a gel dryer and exposed to X-ray films.

### ***cDNA array analysis.***

RNA obtained from MF10F cells treated with 50ug of r-hCG for 24 hours was hybridized to cDNA filter arrays containing 1,176 human genes (Clontech Human Cancer 1.2 array) using the procedures described by the manufacturer.

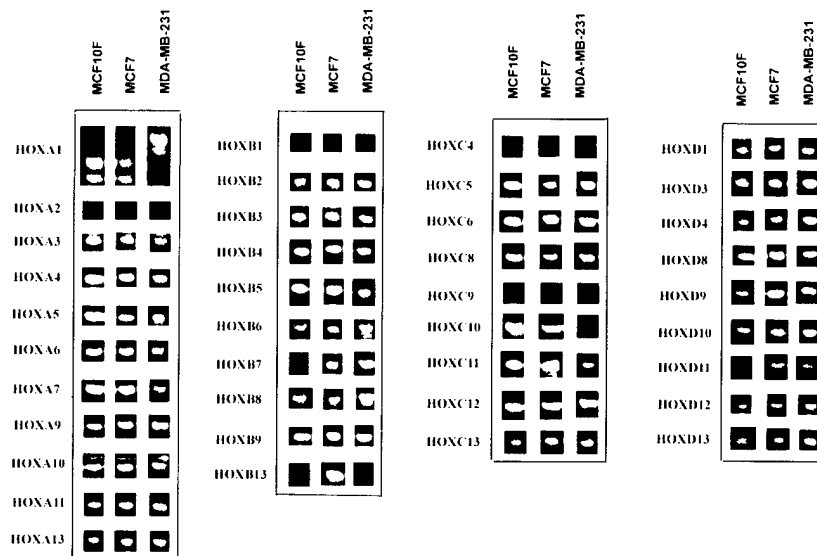
**Table 1: Gene specific primers for analysis of HOX genes expression by RT-PCR**

Gene	5' Primer	3' Primer	T <sub>0</sub> C	Size(bp)
HOXA1	5' ATGAACCTCTTCTTCTGGAATA 3'	5' CGTACTCTCCAACCTTCC 3'	57	452; 655; 1121
HOXA2	5' ATCCCTGGATGAAGGAGAAGAAGGC3'	5' ATTGGGAGCCTGCTGTTGAGAGAGC3'	60	487
HOXA3	5' GAAGAATTCAGGAAGCGCC3'	5' TTCCAGCAACCAAGATTGC3'	56	223
HOXA4	5' TGTACCCCTGGATGAAGAAGATCC3'	5' CATTCTCCGGTTCTGAAACCAGATC3'	60	224
HOXA5	5' TGC GCAAGCTGCACATAAGTCATG3'	5' TTGAAGTGGAACTCCTTCTCCAGC3'	60	112
HOXA6	5' GATGCAGCGCATGAACTCCTGCG3'	5' TGGGCTGCGTGGAATTGATGAGC3'	60	250
HOXA7	5' GATCTGGAGAAGGAGTTCCA3'	5' CTTTCTTCCACTTCATACGA3'	55	134
HOXA9	5' CAGCCAACTGGCTTCATGCG3'	5' CACTCGTCTTTTGCTCGGTC3'	55	229
HOXA10	5' AGAGCAGCAAAGCCTCGCCGGAGAAG3'	5' GGACGCTGCGGCTAATCTCTAGGCG3'	65	198
HOXA11	5' ACCCGCAAAAAGCGCTGC3'	5' GAGCTAGCAGGACAGTTG3'	55	114
HOXA13	5' GGGAGAAAGAAGCGCGTG3'	5' CGTCGTGGCTGATATCCG3'	55	114
HOXB1	5' CCTTCTTAGAGTACCCACTCTG3'	5' GCATCTCCAGCTGCCTCCTT3'	55	826
HOXB2	5' TCCTCCTTTCGAGCAAACCTTCC3'	5' AGTGAATTCTTCTCCAGTTCC3'	55	353
HOXB3	5' AGTACAAGAAGGACCAGAAGGC3'	5' TGGAGTGTTAAGGCGTTCATG3'	60	120
HOXB4	5' GTGCAAAGAGCCCCGTCGTCTACC3'	5' CGTGTCAGGTAGCGGTTGTAGTG3'	60	161
HOXB5	5' GTTCCACTTCAACCGCTACC3'	5' TGTCTTCTTCCACTTCATGC3'	58	122
HOXB6	5' GGCGAGGCCGCCAGACATAC3'	5' ACTCGGCCTGTTTTCTTCC3'	55	236
HOXB7	5' AGAGTAACTTCCGGATCTA3'	5' TCTGCTTCAGCCCTGTCTT3'	55	274
HOXB8	5' AGCCTCCTTGTCGAATTG3'	5' GTAACAATTGCCACAGC3'	54	300
HOXB9	5' GAGCAGGGCAAAGAGTAA3'	5' CTTTCTCCTGACACCTAG3'	54	250
HOXB13	5' CTGGAACAGCCAGATGTGTT3'	5' TTGGCGAGAACCTTCTTCTC3'	60	300
HOXC4	5' CAGTATAGCTGCACCAGTCTCCAGG3'	5' GATCTGCCTCTCAGAGAGGCACAG3'	60	406
HOXC5	5' TGGATGACCAAACAGCACATGAGC3'	5' CAAGTTGTTGGCGATCTCTATGCG3'	60	149
HOXC6	5' CACCTTAGGACATAACACACAGACC3'	5' CACTTCATCCGGCGGTTCTGGAACC3'	60	317
HOXC8	5' CCACGTCCAAGACTTCTTCCACGGC3'	5' CACTTCATCCTTCGATTCTGAAACC3'	58	449
HOXC9	5' TGGTTTCAGACCCGGAGGATCAAG3'	5' GGAAGAGAACGCAGTTTCTCTCC3'	58	436
HOXC10	5' CTACCGCCTGGAACAACCTGTTGG3'	5' ATGGTCTTGCTAATCTCCAGGCG3'	58	662
HOXC11	5' AGAAGCTCGTGCCCTTATTCG3'	5' ATACTGCAGCCGGTCTCTGC3'	57	191
HOXC12	5' AAAGAAGGCGCAAGCCGTATTCGAAG3'	5' AGACGTTGCTCCCTCAGCAGAAGTC3'	58	192
HOXC13	5' TGCCCTATACCAAGGTGCAG3'	5' TAGATTTGCTGACCACCTTT3'	60	173
HOXD1	5' AATCTGGTTCCAGAACCGC3'	5' ATACAAGCTTGCATGCCTGC3'	58	198
HOXD3	5' CATCAGCAAGCAGATCTTCC3'	5' AGCGGTTGAAGTGAATTCC3'	58	187
HOXD4	5' TGGATGAAGAAGGTGCACG3'	5' ACTTCATCCTCCGGTTCTGG3'	58	220
HOXD8	5' AATCAGCAGCTCCTGGTCGACG3'	5' TGTCTTCTCCAGCTCTTGG3'	56	279
HOXD9	5' GATGAGCAAGGAGAAATGCC3'	5' AGTCGCTGGAGAGTTTCTGG3'	56	104
HOXD10	5' TACTCCAACCAGCAATTGGC3'	5' CTCGGATTTCGATTCTCTCGG3'	57	233
HOXD11	5' ATGCTCAACCTCACTGACCG3'	5' AGTTCCACAGAGAAGAGGCG3'	58	230
HOXD12	5' CCAGCTTCAAGGAAGACACC3'	5' CCGCTTCTTCTTCATACGC3'	60	333
HOXD13	5' AGAATGGAGAACGAGTATGCC3'	5' CGGTTCTGAAACCAAATGG3'	55	114

## 6-B-Results

### 6-B-i- Survey of Class I HOX Gene Expression in Human Breast Epithelial Cell Lines.

We analyzed the expression pattern of the four HOX gene clusters in MCF-10F, MCF-7 and MDA-MB-231 cells. The RT-PCR amplified fragments of the expected size were obtained for each gene by using the total cellular RNA from three original cell lines and two specific primers (See table 1 for details). Of the thirty-nine class I homeobox genes, only four, HOXA2, B1, C4, and C9, were silent or not detected in all of the three cell lines by our RT-PCR method. HOXB7 was silent in MCF-10F cells and C10 in MDA-MB-231 cells, while B13 expressed only in MCF-7 cells (Figure 1). The expression pattern is schematically summarized in Figure 2 showing 33 out of the 39 cluster I homeobox genes are expressed in the immortalized human breast epithelial cells MCF-10F, 35 in human breast cancer ER-positive cells MCF-7 and 36 in human breast cancer ER-negative cells MDA-MB-231



**Figure 1.** RT-PCR analysis of the HOXA, B, C and D gene expression in MCF10F, MCF7 and MDA-MB-231 cell lines. Cellular total RNA was isolated from cultured cells. After 40 cycles of amplification with the specific primers, RT-PCR products were electrophoresed on 2% agarose gels. All the amplified products exhibit the expected size.

#### 6-B-ii- r-hCG Modulates Expression of HOX Genes:

To study possible modulation of HOX gene expression by r-hCG, a semi-quantitative RT-PCR approach was used. The total RNA of r-hCG treated and untreated cell lines was screened for differences in HOX gene expression at specific time intervals. Based on this, we observed clear differences in expression of HOXA1, D8, D10, D11 and D13 in MCF-10F cells (Figure 3), HOXA1, B3, B8 and D11 in MCF-7 cells (Figure 4), and HOXA1, C8, D8 and D11 in MDA-MB-231 cells (Figure 5). Minimal or no effects were seen on the expression of other HOX genes (data not shown). Our results showed that after treating the human breast epithelial cells with 5 mcg of r-hCG per ml culture medium for 1, 5, 10, 24, 48 and 96 hours, respectively, in MCF-10F cells, r-hCG rapidly down-regulated all the three transcripts of



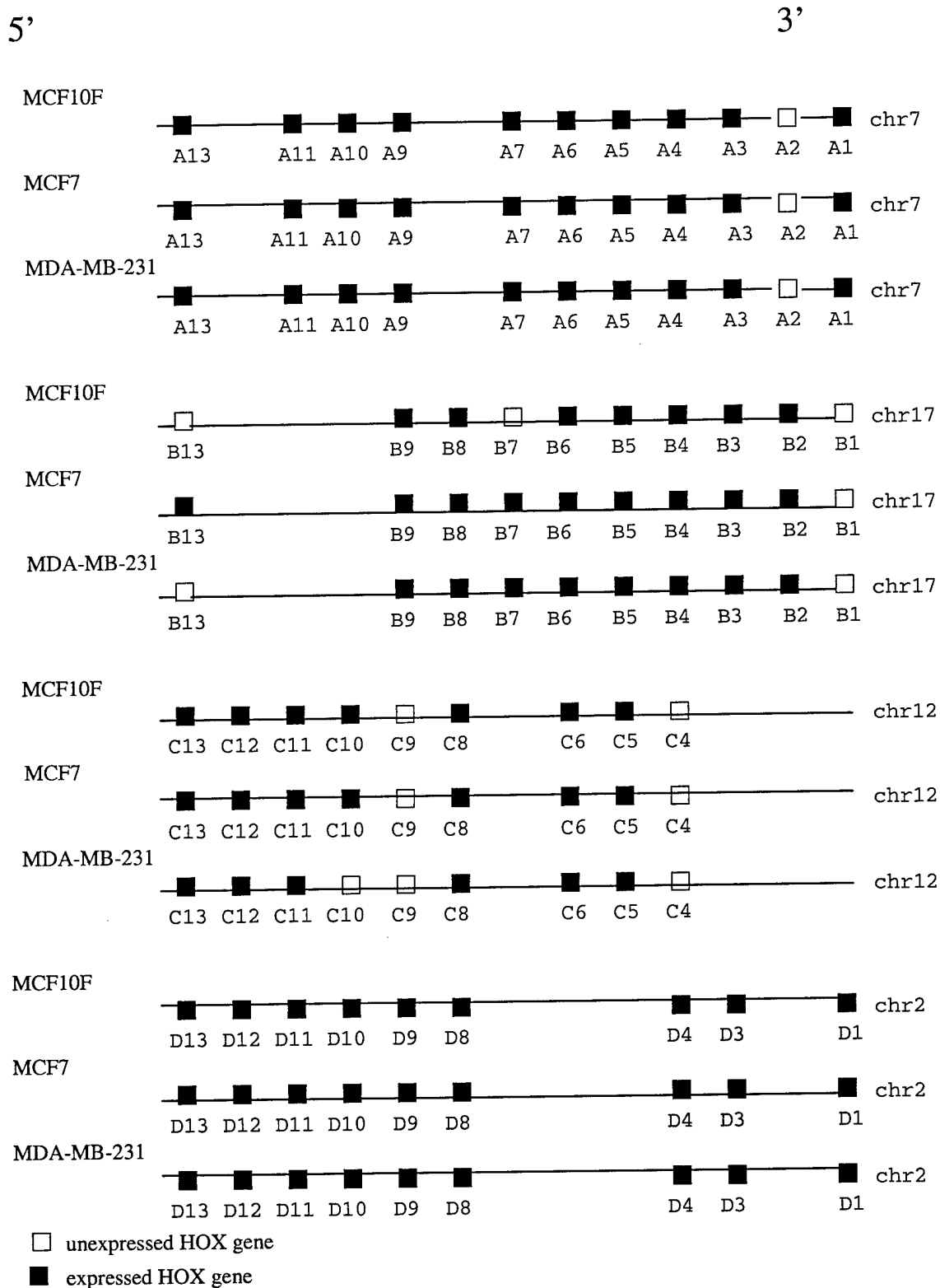


Figure 2: Diagram of HOX gene expression in MCF10F, MCF7 and MDA-MB-231 cells. Homeobox clusters are shown according to their physical position on the chromosomes.

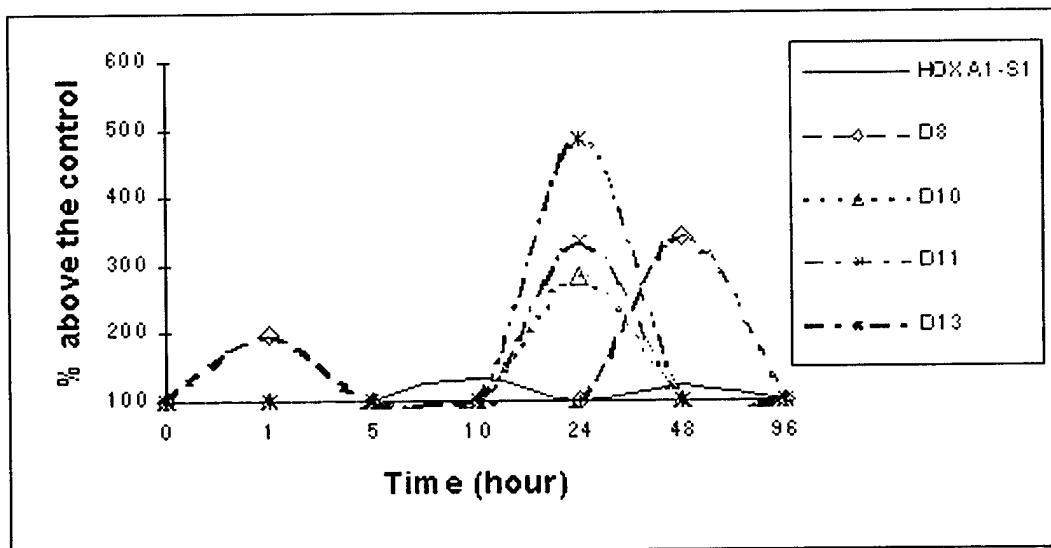
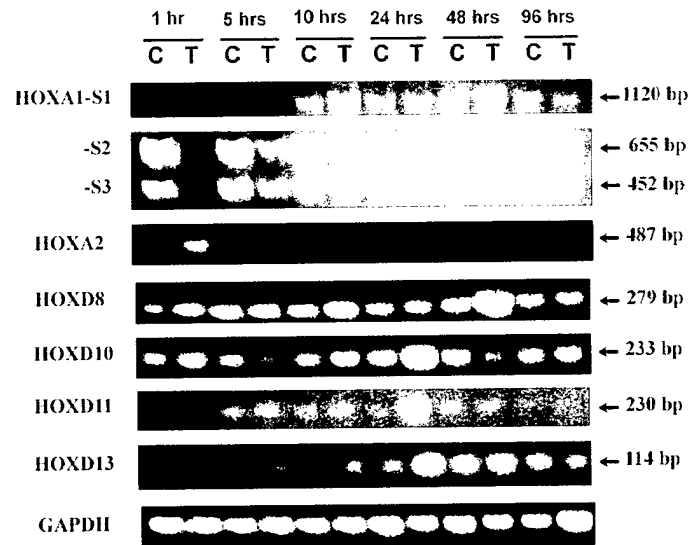


Figure 3: Results of the semi-quantitative RT-PCR analyses with total RNAs from MCF-10F cells treated with r-hCG for 1, 5, 10, 24, 48 and 96 hours, compared to untreated cells and normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. C: control cells; T: hCG-treated cells; S1~S3: alternatively spliced HOXA1 gene.

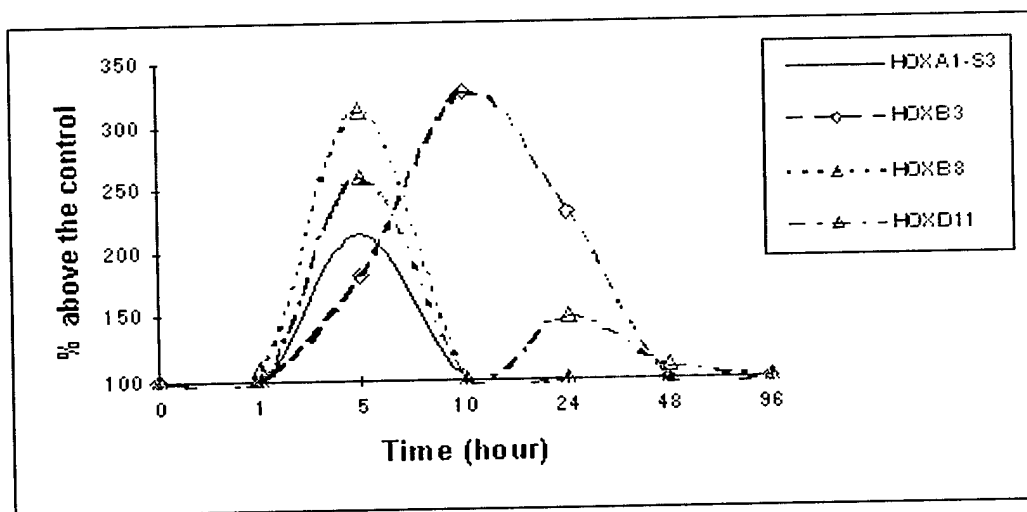
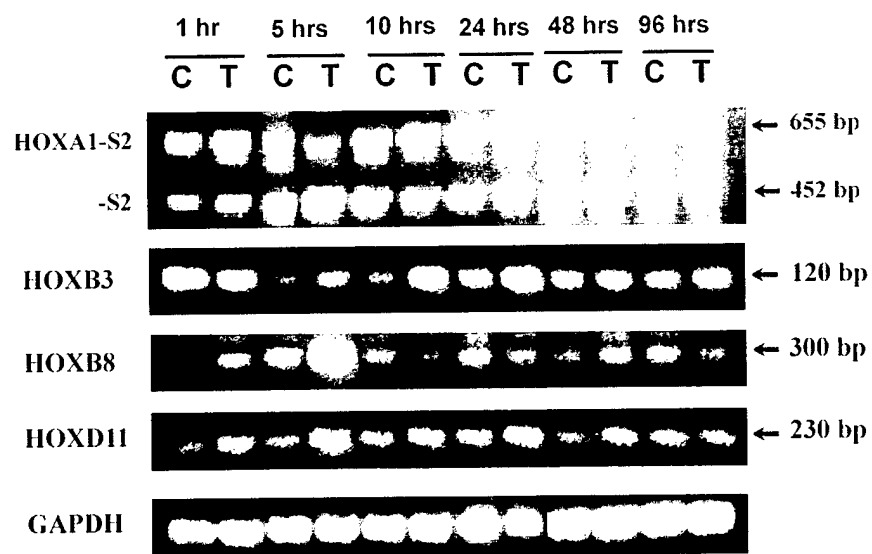


Figure 4: Results of the semi-quantitative RT-PCR analyses with total RNAs from MCF-7 cells treated with r-hCG for 1, 5, 10, 24, 48 and 96 hours, compared to untreated cells and normalized by GAPDH gene. C: control cells; T: hCG-treated cells; S2, S3: alternatively spliced HOXA1 genes.

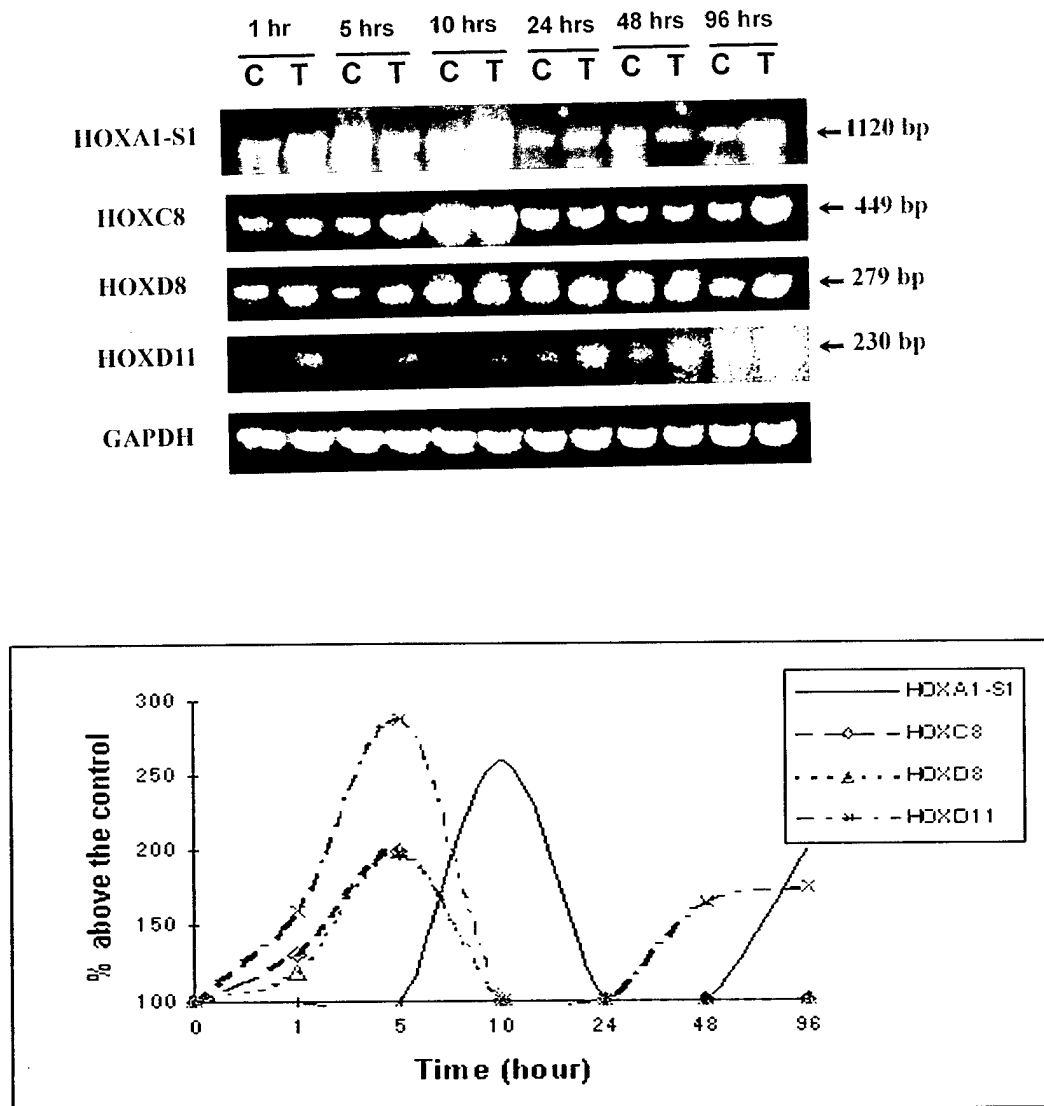


Figure 5: Results of the semi-quantitative RT-PCR analyses with total RNAs from MDA-MB-231 cells treated with r-hCG for 1, 5, 10, 24, 48 and 96 hours, compared to untreated cells and normalized by GAPDH gene. C: control cells; T: hCG-treated cells; S1: alternatively spliced HOXA1 gene.

HOXA1(HOXA1-S1, S2 and -S3) at 1- and 5-hour points, and a 24-hr treatment resulted in up-regulation of HOXD10 (3.8 folds), D11 (4.2 folds) and D13 (5.8 folds), whereas at 48 hours of treatment only D8 was up-regulated by 4.2 folds. In MCF-7 cells, r-hCG treatments of 5 and 10 hours resulted in up-regulation of HOXA1-S3(3.1 folds), B3 (4.2 folds), B8 (4.1 folds) and D11 (3.6 folds). In MDA-MB-231 cells, 5 and 10 hour-treatments up-regulated HOXA1-S1(3.6 folds), C8(2.0 folds, D8 (2.4 folds) and D11 (3.8 folds). A significant finding of this study is that hCG rapidly induces the transient expression of HOXA2, the silent gene in MCF-10F (Figure 2 and 3).

#### 6-B-iii- HOXA1 Divergent Expression and Induction by hCG in Human Breast Epithelial Cells.

There were three fragments of 1121bp, 655bp and 452bp observed on HOXA1 gene amplification. Sequence analysis confirmed that they are respectively belonged to three alternatively spliced transcripts of HOXA1 described by Chariot (1995) (figure 6).

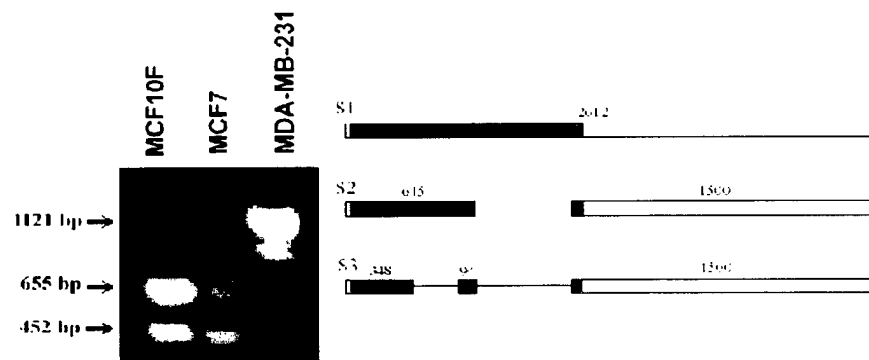


Figure 6. Expression and structures of HOXA1 mRNA isoforms in human breast epithelial cells. Straight lines indicate introns and the black boxes correspond to the detection portions (1121 bp, 655 bp and 452 bp) of alternatively spliced HOXA1 gene(S1-S3) by RT-PCR.

Table 2. hCG modulated genes that encode transcription factors/DNA binding protein

Gene/Protein name	Accession Number	Fold	Classification/Function
c-myc	P01106	1.5	Oncogene & tumor suppressor, Other transcription protein.
Puf	P22392	2.02	Transactivating factor; interaction with an element located upstream from the c-myc gene.
RhoE	P52199	2.00	Oncogene & tumor suppressor; Rho-related GTP-binding protein.
EIF-1	P32519	3.12	Transcription activators and repressor; regulating differential gene expression.
GSPT1(GST1-HS)	P15170	-5.0	A GTP-binding protein essential for the G1-to S-phase transition of the cell cycle.
AP-1	P05412	-2.0	Transcription activator and repressor.
DNA binding protein CPBP	Q99612	-3.0	DNA binding protein involved in the regulation of TATA box-less gene
G rich sequence factor 1(GRSF1)	Q12849	-3.10	mRNA binding protein involved in mRNA processing

#### 6-B-iv- r-hCG and HOXA2 Inhibits AP-1

To explore if hCG alter the expression of AP-1, we performed the electrophoretic mobility shift assay (EMSA) using cell extracts of MCF10 F cells treated by 1, 12 and 24 hours with 50ug of r-hCG as it is

depicted in figure 7. There is significant reduction in the protein binding expression. In order to determine if HOXA2 could be involved in this pathway, we examined the effect of HOXA2 in the expression of AP-1, since the expression of HOXA2, the silent gene in MCF10F cells was induced by hCG treatment in vitro. Using EMSA we observed a higher level of AP-1 activation by TNF in MCF-10F cells (Figure 8). However, after transient transfection with HOXA2 cDNA expression construct, the AP-1 binding activity was significantly decreased. Indicating that the effect of r-hCG on AP1 expression is modulated by HOX A2. This confirms the decrease in the level of AP-1 observed in MCF10F cells treated with r-hCG by EMSA (Figure 7) as well as in the downregulated genes observed in a c-DNA array membrane (Table 2).

#### ***6-B-v.- Effect of r-hCG on differential gene expression.***

This study was performed using the RNA obtained from MCF 10F cells treated with hCG with 50ug of hCG for 24 hours and hybridized to cDNA array membranes that contain 1,176 human genes (Clontech Human Cancer 1,2 array). The expression profiles of MCF-10F cells revealed that exogenous hCG significantly increased the mRNA levels of four genes encoding cmyc, Puf, RhoE and EIF-1 and depress the expression of GSPT1, AP1, DNA binding protein CPBP and G-rich sequence factor (Table 2).

#### ***6-C-Discussion***

In the present work we report for the first time that 33 out of the 39 cluster I homeobox genes are expressed in the immortalized human breast epithelial cells MCF-10F, 35 in human breast cancer ER-positive cells MCF-7 and 36 in human breast cancer ER-negative cells MDA-MB-231. We also show that there is a differential expression of alternatively spliced transcripts of HOXA1 in these three HBECs and that hCG modulates the expression of some HOX genes. In MCF-10F cells, r-hCG rapidly down-regulated all the three transcripts of HOXA1 (HOXA1-S1, S2 and -S3) at 1- and 5-hour points, and transiently induced expression of HOXA2, the silent gene, at very early stage. In addition, exogenous r-hCG could increase the expression levels of HOXD8, D10, D11 and D13 genes. In MCF-7 cells, r-hCG treatment resulted in up-regulation of HOXA1-S3, B3, B8 and D11 genes. In MDA-MB-231 cells, r-hCG treatment up-regulated the expression of HOXA1-S1, C8, D8 and D11 genes. Using a cDNA array filter containing 1,250 genes we identify that r-hCG was able to modulate the expression of eight transcription factors. AP1 was among those transcription factors that was depressed in MCF10-F cells treated with r-hCG and confirmed by electrophoresis mobility shift assay. Furthermore, we have demonstrated that this effect was mediated by HOXA2, because when HOX A2 were transiently expressed in MCF10F cells AP-1 was inhibited.

The RT-PCR amplification of HOX gene transcripts with primers that recognize specific HOX genes has been widely used for analyzing the expression level of HOX genes in various human cancer or normal cells [18,19]. Despite extensive literature on the role in embryonic and fetal development, HOX gene expression in adult cells has been reported only recently in a few tissues, including kidney, intestine, testis, colon, and the mouse mammary gland [20,21,22,23]. Our study provides evidence for the first time that 33 out of the 39 cluster I homeobox genes are expressed in the immortalized human breast epithelial cells MCF-10F, 35 in human breast cancer ER-positive cells MCF-7 and 36 in human breast cancer ER-negative cells MDA-MB-231. Similar number has been detected in other human adult tissues/cells such as the colon (29/38), kidney (30/38) and cervix keratinocytes (34/39) [3,4,19]. Hox B7 was absent in MCF10F but it was expressed in the neoplastic cells, which may suggest an association with oncogenic transformation in human breast epithelium. But, in mouse mammary gland Hox b7 may play a role in

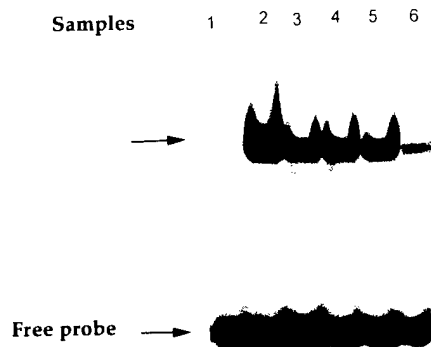


Figure 7: EMSA were performed using  $3\mu\text{g}$  of nuclear extracts from MCF-10F cells treated with  $50\mu\text{g}$  of r-hCG with. Lane 1, no extract (negative control); lane 2, HeLa extract (positive control); lane 3, MCF-10F cells; lane 4, MCF-10F cells with 1-hr-treatment of r-hCG; lane 5, MCF-10F cells with 12-hr-treatment of r-hCG; lane 6, MCF-10F 24 hour treatment with r-hCG.

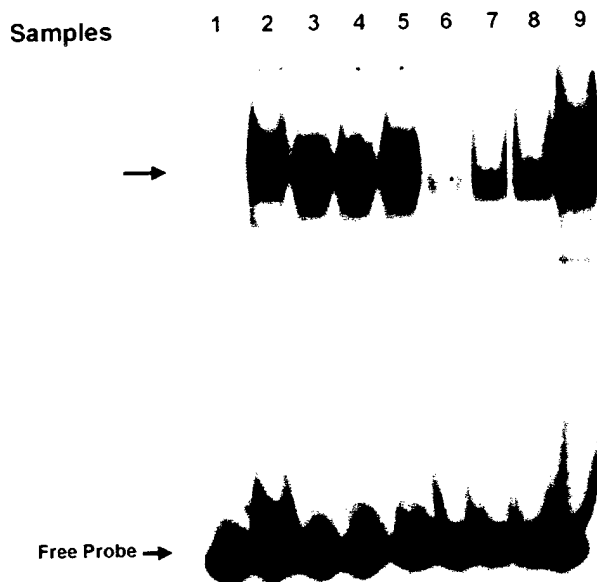


Figure 8. EMSA were performed using  $3\mu\text{g}$  of nuclear extracts from MCF-10F cells transfected with or without pCMV-HOXA2 plasmid. Lane 1, no extract (negative control); lane 2, HeLa extract (positive control); lane 3, MCF-10F cells; lane 4, MCF-10F cells with 1-hr-treatment of RANK ligand; lane 5, MCF-10F cells with 12-hr-treatment of RANK ligand; lane 6, MCF-10F cells transfection with pCMV-HOXA2 plasmid (10F-A2+); lane 7, 10F-A2+ cells with 1-hr-treatment of RANK ligand; lane 8, 10F-A2+ cells with 12-hr-treatment of RANK ligand; lane 9, 10F-A2+ cells with 24-hr-treatment of RANK ligand. A), AP-1 EMSA; B), NF- $\kappa$ B EMSA

remodeling and in reestablishing ductal branching [22]. The transduction of HOXB7 has been shown to induce bFGF expression and alter growth characteristic of human breast cancer cells [23]. Altogether these data suggest that the expression of most class I HOX genes in human breast epithelial cells may play some role in the control of differentiation and neoplastic transformation of these cells.

The types of genes activated vary with the biological characteristics of the cells, suggesting that the expression and regulation of HOX genes were cell type-specific. The exact mechanism of its actions, however, is still poorly understood. Some homeobox genes have been proven to be an early response genes to the activin that is composed of two  $\beta$ -strains of inhibin [24]. Our previous study found that the effect of hCG in inhibition of rat mammary tumorigenesis was associated with increased expression of inhibin [25]. HOX gene expression, in general, affects cell growth, differentiation, and fate [26]. There were three fragments of 1121bp, 655bp and 452bp observed on HOXA1 gene amplification. Sequence analysis confirmed that they belonged to three alternatively spliced transcripts of HOXA1 described by Chariot [27]. In addition to its role in development, Hox-a1 is involved in murine cellular transformation and/or mammary gland tumorigenesis [7,23]. The data we presented here indicates that r-hCG down-regulated all the three transcripts of HOXA1 at very early stage in the immortalized human breast epithelial cells MCF-10F, whereas HOXA1-S1, the largest transcript, as well as HOXA1-S3, the smallest transcript, were up-regulated in the cancer cell lines MDA-MB-231 and MCF-7, respectively. The relationship between HOXA1 expression and human breast cancer lesions has been reported [9]. It had been found that the HOXA1 transcripts could be induced by retinoic acid in MCF-7 cells [27]. Our results, combined with these data, suggest that HOXA1 may play a role in breast epithelial differentiation and neoplastic transformation.

A significant finding of this study is that hCG rapidly induces the transient expression of HOXA2, the silent gene in MCF-10F. So far, fewer data have been available for understanding the biological function of HOXA2 in adult. Its regulation mechanism is totally unclear. To explore if hCG effect is mediated by some of the HOX genes, we performed the electrophoretic mobility shift assay (EMSA) for AP-1 transcription factor using cell extracts of MCF10 F cells treated by 1, 12 and 24 hours with 50ug of r-hCG, and was observed a significant reduction in the protein binding expression. We further demonstrated that HOXA2 is involved in the modulation of AP-1, since using EMSA we observed a higher level of AP-1 activation by TNF were observed in MCF-10F cells. However, after transient transfection with HOXA2 cDNA expression construct, the AP-1 binding activity was significantly decreased. Indicating that the effect of r-hCG on AP1 expression is modulated by HOX A2. This confirms the decrease in the level of AP-1 observed in MCF10F cells treated with r-hCG by EMSA as well as in the downregulated genes observed in a c-DNA array membrane. Whereas the other transcription factors/ DNA binding proteins are also of great interest [28-42] for our understanding the action of hCG we have concentrated in this work in the AP-1. The activation of AP-1 is mediated through the activation of a stress-activated protein kinase called c-jun N-terminal kinase (JNK) [43]. E6/E7 immortalized human keratinocytes transfected with dominant negative *c-jun* TAM67 showed a reduction in the elevated AP-1 activities seen with progression and suppression of tumor phenotype [44]. In addition, cotransfection of MCF-7 cells with a c-jun expression vector and the EGFR promoter reporter resulted in a 7-fold increase in promoter activity [45], and an elevated level of c-jun activation related to poorer quality and shortened duration of endocrine response in estrogen-receptor-positive breast cancer patients [46]. Recently, transfection of JB6 cells with *pdc4*, a novel transformation suppressor gene, resulted in inhibition of AP-1 transactivation [47]. Our data indicate that the over-expression of HOXA2 protein alone was not sufficient to block the TNF-induced AP-1 activation, suggesting there are other factors involved, since hCG has been reported to be a suppressor on TNF-induced AP-1 activation [14], probably by activation of HOXA2. Altogether our data have shown that the majority of HOX genes are active in human breast epithelial cells and they have a divergent expression pattern that is modulated by exogenous hCG. This not only implies a role of HOX genes in the process of differentiation and transformation of HBEC, but also identifies hCG as a novel regulator in the complicated HOX network.



## 7.-KEY RESEARCH ACCOMPLISHMENTS

A) **A comprehensive survey of class I homeobox genes expression in HBECs.** Our study evidences for the first time that 33 out of the 39 cluster I homeobox genes are expression in the immortalized human breast epithelial cells MCF-10F, 35 in human breast cancer ER-positive cells MCF-7 and 36 in human breast cancer ER-negative cells MDA-MB-231.

B) **Differential expression of alternatively spliced transcripts of HOXA1 in these three HBECs.** Since HOXA1 was assumed to be a important factor that regulate breast epithelial cell differentiation, these data elucidated its crucial role in establishment of the human breast cancer cell phenotype.

C) **The first evidence that hCG modulate expression of some HOX genes.** The information provides prospect on the mechanism of action of hCG on mammary cell differentiation and furthermore the effect of this hormone in breast cancer prevention.

i-In MCF-10F cells, r-hCG rapidly down-regulated all the three transcripts of HOXA1(HOXA1-S1, S2 and -S3) at 1- and 5-hour points, and transiently induced expression of HOXA2, the silent gene, at very early stage. In addition, exogenous r-hCG could increase the expression levels of HOXD8, D10, D11 and D13 genes

ii- In MCF-7 cells, r-hCG treatment resulted in up-regulation of HOXA1-S3, B3, B8 and D11 genes.

iii.-In MDA-MB-231 cells, r-hCG treatment up-regulated the expression of HOXA1-S1, C8, D8 and D11 genes.

D) Using a **cDNA array technique** we have been able to demonstrate that hCG activates the expression of transcription factors that in turn may regulates genes responsible for critical events in cell differentiation such as, DNA repair, apoptosis and cell transformation.

E). A significant finding of this study is that hCG rapidly induces the transient expression of HOXA2, the silent gene in MCF-10F. We further demonstrated that HOXA2 is involved in the modulation of AP-1, since using EMSA we observed a higher level of AP-1 activation by TNF were observed in MCF-10F cells. However, after transient transfection with HOXA2 cDNA expression construct, the AP-1 binding activity was significantly decreased. Indicating that the effect of r-hCG on AP1 expression is modulated by HOX A2.

## 8.-REPORTABLE OUTCOMES

### A) Abstract

Russo, J., Lareef, M.H., Russo, I.H., and Jiang, X. Modulation of Hox gene expression in human breast epithelial cells by human chorionic gonadotropin. Proc. Am. Assoc. Cancer Res. 42:2649a, 2001

B) *Manuscript in preparation*

Jiang, X., Russo, I.H., Lareef, M.H., and Russo, J. hCG regulate the expression of HOX genes in human breast epithelial cells.

*C) Application for an IDEA award*

Presentation of an IDEA award to the Department of defense for elucidating the role of HOXA1 gene in breast cancer prevention.

## 9.-CONCLUSIONS

We have shown that the majority of HOX genes are active in human breast epithelial cells and they have a divergent expression pattern that is modulated by exogenous hCG. This not only implies a role of HOX genes in the process of differentiation and transformation of HBEC, but also identifies hCG as a novel regulator in the complicated HOX network.

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## **11.-PERSONNEL INVOLVED IN THIS PROJECT**

**Jose Russo, MD** is the principal investigator of this project and he has directed all the phases of designing, interpreting and writing the final outcome of this project.

**Xiaoshang Jiang, Ph.D.** is a postdoctoral associate that has conducted most of the work described in this report.

**TITLE:** Homeobox genes Mediates the Biological Functions of Human Chorionic Gonadotropin (hCG) in Human Breast Epithelial Cells

**PRINCIPAL INVESTIGATOR:** Jose Russo, M.D.

**APPENDIX:**

**Abstract**

Russo, J., Lareef, M.H., Russo, I.H., and Jiang, X. Modulation of Hox gene expression in human breast epithelial cells by human chorionic gonadotropin. Proc. Am. Assoc. Cancer Res. 42:2649a, 2001



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**Modulation of HOX Gene Expression in Human Breast Epithelial Cells by Human Chorionic Gonadotropin.** X. Jiang, I.H. Russo, M.H. Lareef, and J. Russo. *Breast Cancer Research Laboratory, Fox Chase Cancer Center, Philadelphia, PA.*

The placental hormone human chorionic gonadotropin (hCG) stimulates mammary gland differentiation and inhibits tumor progression. Because class I homeobox genes (Hox) are involved in mouse mammary gland development and in breast cancer, we hypothesized that hCG's effect is mediated by HOX gene activation. Reverse transcription-polymerase chain reaction (RT-PCR) was used for studying the expression of 39 known HOX genes in MCF-10F, MCF-7, and MDA-MB-231, an immortal and two cancer breast cell lines. Thirty five of the 39 class I homeobox genes analyzed were expressed by the three cell lines; four, HOXA2, B1, C4, and C9 were not detected in any of them. Semi-quantitative RT-PCR technique was used for studying the expression patterns of these genes in cells treated with 5 µg of recombinant hCG (r-hCG)/ml for 1, 5, 10, 24, 48 and 96 hrs. In MCF-10F cells the 24-hr treatment up-regulated HOXD10 (3.8 folds), D11 (4.2 folds), and D13 (5.8 folds), whereas at 48 hrs of treatment only D8 was upregulated 4.2 folds. The silent gene HOXA2 was transiently detected at 1 hr of treatment. In MCF-7 cells, r-hCG treatments of 10 and 24 hrs resulted in upregulation of HOXB3 (2.5 folds), B8 (1.5 folds) and D8 (2.2 folds). In MDA-MB-231 cells, 1 and 5 hr-treatments upregulated HOXC8 (2.0 folds), C12 (2.0 folds), D8 (2.4 folds) and D11 (3.8 folds). Although HOXD8 was activated in the three cell lines, this effect varied with the time of treatment. The types of genes activated also varied in normal immortalized and in cancer cell lines, indicating that hCG plays a role in the regulation of HOX genes in human breast epithelial cells, but the effect is modulated by the biological characteristics of the cells. (Supported by grants DAMD 17-19-1-9182 and DAMD 17-00-1-0249).

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